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54 Plasmid vectors for expression in escherichia coli and/or bacillus subtilis.

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EP-A- 0 036 259
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NUCLEIC ACIDS RESEARCH, vol. 5, no. 12,
December 1978, Oxford, GB, E. BECK et al. "Nucleotide
sequence of bacteriophage fd DNA" pages 4495-4503.

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Description

The present invention relates to novel plasmid vectors for expression in *Escherichia coli* and/or *Bacillus subtilis*, in which the gene which codes for a heterologous protein is placed under the control of regulating sequences which permit the organisms transformed with the abovementioned plasmids to express the heterologous protein in high yields.

In particular, the present invention relates to the plasmid vectors pSM146 and pSM147 for expression in *E. coli*, in which the gene which codes for a heterologous protein is placed under the control of the terminator sequence of the phage fd of *E. coli* and of the pE194 erythromycin promoter modified by replacing the -35 region with segments of synthetic DNA.

The present invention further relates to the plasmid vector pSM143 for expression in *E. coli* and *B. subtilis* and useful for the formation of the plasmid vectors pSM146 and pSM147.

Recent developments in the field of genetic engineering have made it possible to prepare specific proteins by means of a process which includes the formation of a plasmid vector containing a heterologous gene which codes for a specific protein, to introduce the said vectors into host organisms, to isolate the said transformed organisms and to cultivate the same in culture media which are suitable for the purpose of obtaining the expression of the coded protein by the heterologous gene.

The expression "heterologous gene" designates a DNA which codes for a protein which is generally not produced by the host organisms which are used and which is referred to as a heterologous protein.

The term "expression" is used to indicate the process by which a protein is synthesized by an organism.

The said process takes place in two phases. The first, referred to as transcription, consists in the transfer of the genetic information from the DNA to the messenger RNA (mRNA). The second phase, referred to as translation, concerns the transfer of the information from the mRNA to the protein.

The transcription of the DNA into mRNA takes place by means of the enzyme RNA polymerase, and commences at a specific point of the DNA which is located in the vicinity of the sequence referred to as the promoter, and terminates at another well specified point, in the vicinity of the region of DNA defined as the terminator.

Because a specific heterologous protein is synthesized within a host cell, it is necessary to place the structural gene of the said protein under the control of specific sequences, i.e. the promoter, the terminator and the ribosomal binding site (RBS), which are recognized by the host organism and which permit the mRNA polymerase to transcribe the DNA into mRNA and permit the ribosomes and relevant enzymes to translate the mRNA into proteins.

It is well known that it is possible to obtain enhanced expression of a heterologous protein, by placing the structural gene of the protein under the control of a "strong" promoter.

A strong promoter is understood to be a promoter which exhibits specific sequences of nucleotide bases.

According to M. Rosenberg and D. Court (Ann. Rev. Genet. 13 (1979), 319-353) the strong promoter sequence which is considered to be optimal is formed in the -35 region by the bases TTGACA and the -10 region by the bases TATAAT, with a spacer of 17-18 nucleotides between the two.

It is also well known that the maintenance of the said strong promoters on the plasmid vectors is dependent upon the simultaneous presence of a terminator which is as strong (R. Genko et al. PNAS 78, (1981) 4936-4940).

The terminator in fact determines the detachment of the RNA polymerase from the DNA molecule, thus preventing this from interfering with the processes of replication of the plasmid DNA. The absence of the terminator would cause a reduction in the number of copies of plasmids per cell and, consequently, the loss thereof from the cell progeny. Just like the promoters, the terminators are also defined, depending upon their sequence, as more or less strong, thus having a capacity to bring about the release of the RNA polymerase in a more or less effective manner.

Thus, in order to obtain good plasmid stability, a large number of copies of plasmids per cell and, in consequence, efficient expression of the heterologous protein, it is necessary that a strong promoter should be associated with an equally strong terminator. In the literature, the terminator of the phage fd of *E. coli* (E. Beck et al., Nucleic Acids Res. (1978) 5, 4495-4503) is described as a particularly strong terminator. We have now found that, by modifying the erythromycin promoter of pE194, a plasmid present in *B. subtilis* BGSC 1E7, by replacing the -35 region with suitably designed segments of synthetic DNA, it is possible to obtain promoters which are stronger than that at the outset.

The said promoters are maintained in a stable state at the plasmid level by virtue of the simultaneous presence of the terminating sequence of the phage fd of *E. coli*, and the microorganisms transformed with the plasmid vectors containing the said promoters are capable of expressing the heterologous proteins with high efficiency.

In accordance with this, the scope of the present invention is constituted by novel plasmid vectors for expression in *E. coli* and/or *B. subtilis*, in which the gene which codes for a heterologous protein is placed under the control of regulating sequences which permit the organisms transformed with the abovementioned plasmids to express the heterologous protein in high yields.

In particular, the scope of the present invention is constituted by the plasmid vectors pSM146 and pSM147 for expression in *E. coli*, in which the heterologous gene which codes for a specific protein is placed under the control of the terminator sequence of phage fd of *E. coli* and of the pE194 erythromycin promoter modified by replacing the -35 region with segments of synthetic DNA.

The scope of the present invention is furthermore constituted by the plasmid vector pSM143 for

expression in *E. coli* and *B. subtilis* and useful for the formation of the plasmid vectors pSM146 and pSM147.

The plasmid vector pSM143 may also be used for the formation of other plasmid vectors for expression, by replacing the region of the promoter and of the terminator with novel segments of synthetic DNA which code for other promoters and terminators.

According to the present invention, the segments of synthetic DNA in pSM146 and pSM147 exhibit the following sequences respectively:

5' CTG TTTTGTGCAAT³

3' TCGAGAC AAAAAACAGTTAGATC⁵

and

5' CTG TTTTGTGCAACAACCTTTT³

3' TCGAGAC AAAAAACAGTTGTTGAAAAA-
GATC⁵

The said segments are synthesized according to well known techniques (J.H. Van Boom (1975) *J. Biol. Chem.* 250, 4592 - R.L. Letsinger et al. (1975) *J. Amer. Chem. Soc.* 97, 32).

The plasmid vectors pSM146 and pSM147 introduced into cells of *E. coli* HB101 are maintained in a stable state and impart to the cells transformed in this manner a resistance both to ampicillin and to kanamycin up to concentrations of 100 µg/ml and 15 µg/ml respectively.

The cells of *E. coli* transformed in this manner and cultivated in a suitable culture medium are capable of expressing the heterologous proteins in high yields. In accordance with the present invention, in pSM143, pSM146 and pSM147, the gene which is placed under the control of the promoter-terminator sequences employed by us is that of the β-lactamase of pBR322 of *E. coli* (cf. J.G. Sutcliffe *Proc. Natl. Acad. Sci. USA* 75, 3737 (1978)).

Nevertheless, the selection of the gene to be cloned is not critical in relation to the invention, and use may be made of heterologous genes which code for other proteins such as, for example, human and animal hormones, proteins for use in foods, enzymes, etc.

According to the present invention, the plasmid vectors pSM146 and pSM147 are obtained from the plasmid pSM143 by replacing the SstI-XbaI region, having 740 base pairs, with the segments of synthetic DNA which are defined by the sequences set forth above.

The promoter sequences of pSM143 has a single SstI restriction site between the -35 region and the -10 region, and a single XbaI site upstream of the -35 region.

It is thus possible to replace the small segment (740 base pairs) carrying the promoter after digestion of the pSM143 with the specific restriction enzymes SstI and XbaI.

In accordance with the present invention, the plasmid pSM143 is obtained by a process which includes:

a. The formation, from the plasmid pSM110, of the plasmid pSM116, which exhibits a BamHI site in place of the PvuII site,

b. The formation, from the pSM116, of the plasmid

pSM131, which exhibits, downstream of the origin of replication, two restriction sites XhoI and BglII, which are useful for cloning,

c. The insertion into the plasmid pUC8 of the HindIII fragment containing the terminator of the phage of *E. coli* fd, and isolation of the plasmid pSM138,

d. The isolation of the terminator of the phage fd from the plasmid pSM138 after digestion with the restriction enzyme BamHI,

e. The insertion of the BamHI fragment containing the terminator of the phage fd into the BglII site of the plasmid pSM131 and isolation of the plasmid pSM137,

f. The introduction of a HindIII site downstream of the structural gene of β-lactamase by means of in vitro mutation of the plasmid pSM116, and isolation of the plasmid pSM141,

g. The ligation of the HindIII fragment of pSM141 containing the gene of β-lactamase with the HindIII fragment of 3500 base pairs of the plasmid pSM137 after digestion of the two plasmids with HindIII, and isolation of the plasmid pSM142,

h. The ligation of the BamHI-EcoRI fragment of the plasmid pSM142 with the plasmid pSM112 after digestion of the two plasmids with the restriction enzymes BamHI and EcoRI, and isolation of the plasmid pSM143.

In accordance with the present invention, the gene cloned in the plasmid pSM143 is that of the β-lactamase of pBR322 of *E. coli*. Nevertheless, by deleting the EcoRI-HindIII fragment, of 850 base pairs, which contains the β-lactamase gene, it is possible to insert into the vector a gene coding for another protein, which will thus be under the control of regulating sequences (promoter, terminator and RBS) recognized both by *E. coli* and by *B. subtilis*.

According to the present invention, the process for the formation of the plasmid pSM143 provides for the formation of intermediate plasmids starting from the plasmid pSM110, which is obtained according to the description in Italian Patent Application 23.992 A/84 (corresponding European Patent Application is EP-A 186 631) and the restriction map of which is reproduced in Figure 1. The plasmid pSM110 exhibits an EcoRI site located upstream of the ATG of the β-lactamase gene of pBR322 of *E. coli*.

The plasmid pSM143 obtained in this manner exhibits, at the end of the β-lactamase gene, the terminator of the *E. coli* phage fd and, upstream of this, the erythromycin promoter of pE194, the sequence of which, in the -10 region, is in accordance with what is reported by M. Rosenberg et al., while the -35 region differs by two bases, exhibiting the sequence TTCATA.

The plasmid vector pSM143 may furthermore be maintained in a stable condition both in *E. coli* and in *B. subtilis*, in which it confers the resistance to ampicillin up to concentrations of 100 µg/ml and 5 µg/ml, and to kanamycin up to concentrations of 15 µg/ml and 5 µg/ml, respectively.

The plasmid pSM143 is characterized by the restriction map reproduced in Figure 2.

In accordance with the present invention, the formation of the plasmid vectors pSM143, pSM146 and

pSM147 is carried out in accordance with known techniques.

In particular, the digestion of the plasmids is undertaken by suspending the DNA Plasmids in a solution containing 50 mM Tris.HCl (pH 8.1), 50 mM NaCl and 10 mM MgCl₂ in the presence of the restriction enzyme or enzymes suitable for the purpose and under the conditions recommended by the supplier company.

The restriction enzymes used in the present invention were supplied by the companies BRL and BIOLABS. The ligation reaction of the fragments obtained after digestion is carried out by suspending the said fragments in a solution containing 50 mM Tris.HCl, 10 mM MgCl₂, 10 mM dithiothreitol (DTT) and 1 mM ATP in the presence of the enzyme T4 DNA ligase.

The "in vitro mutation" reaction, the purpose of which is to modify in a controlled manner the sequence of base of a specific region of DNA, is undertaken by using the methodology reported by M.I. Zoller et al. in Nucleic Acids Research 10 (1982) 6487-6500.

In order to verify the effect of the various promoters and of the terminator on the expression of the β -lactamase gene of pBR322, the plasmids of the present invention were introduced into *E. coli* HB101 and *B. subtilis* BGSC 1A246 cells. The cells transformed in this manner were respectively cultivated in the following liquid media: LB (Bacto tryptone 10 g, yeast extract 5 g and NaCl 10 g) (DIFCO) and SMS (Splitten minimal salts) with the addition of 0.5% glucose, 50 μ g/ml tryptophan and trace elements, with agitation at 37°C, for 6 hours. At the end of the said period, the β -lactamase activity was determined in the cells for *E. coli*, and both in the cells and in the culture broth for *B. subtilis*, and compared with that obtained by cultivating cells of *B. Subtilis* NRRL B15896 containing the plasmid pSM119 obtained according to what is reported in Italian Application 84/23992. It can be seen from the results obtained that the plasmid pSM143 is stable in *B. subtilis* and *E. coli* and exhibits a β -lactamase activity which is twice as great as that of pSM119, both when introduced into *B. subtilis* and when introduced into *E. coli*.

On the other hand, the plasmids pSM146 and pSM147 prove to be stable only in *E. coli* and exhibit a β -lactamase activity from 6 to 65 times greater than that determined in the strain of *E. coli* containing pSM119.

The strains of *E. coli* HB101 containing the plasmids pSM143, pSM138, pSM146 and pSM147 were disposed on February 22, 1985 at the "American Type Culture Collection" Culture Centre respectively ATCC 53038, ATCC 53037, ATCC 53039 and ATCC 53040.

DESCRIPTION OF THE FIGURES

Figure 1

Shows the restriction map of the plasmid pSM110.

Figure 2

Shows the restriction map of the plasmid pSM143.

Figure 3

Shows the restriction map of the plasmid pSM146 and the sequence of the fragment of synthetic DNA present in the -35 region of the promoter.

Figure 4

Shows the restriction map of the plasmid pSM147 and the sequence of the fragment of synthetic DNA present in the -35 region of the promoter.

Figure 5

Shows the restriction map of the plasmid pSM116.

Figure 6

Shows the restriction map of the plasmid pSM131 and the precise arrangement of the two XhoI linkers.

Figure 7

Shows the restriction map of the plasmid pSM138 and the precise orientation of the HindIII fragment containing the terminator of the *E. coli* phage fd.

Figure 8

Shows the restriction map of the plasmid pSM137 and the arrangement of the terminator of the phage fd before the origin of replication of the plasmid (in the anticlockwise direction).

Figure 9

Shows the restriction map of pSM141 and the stages (in vitro mutation) for preparation thereof.

Figure 10

Shows the restriction map of the plasmid pSM142.

Figure 11

Shows the restriction map of the plasmid pSM112. The experimental examples which follow are illustrative and do not limit the invention.

EXAMPLE 1

Formation of the plasmid pSM116

1 μ g of the plasmid pSM110 is suspended in 20 μ l of a solution containing 50 mM Tris.HCl, pH 8.1, 50 mM NaCl and 10 mM MgCl₂ (solution A) and digested with 1 unit (U) of restriction enzyme PvuII (BIOLABS) at 37°C for 1 hour.

At the end of the reaction the enzyme is inactivated at 65°C for 10 minutes.

1 μ l of the reaction mixture obtained in this manner and 50 ng of phosphorylated BamHI linkers (BIOLABS) are bonded, in the presence of 1 U of enzyme T4 ligase, after addition to 19 μ l of a solution containing 66 mM Tris.HCl, pH 7.6, 6 mM MgCl₂, 10 mM dithiothreitol and 1 mM adenosine triphosphate (ATP) (solution B).

The ligase reaction is conducted at 14°C for 14 hours.

The entire ligase mixture is then used to transform cells of *Escherichia coli* HB101 rendered competent with CaCl₂ by the method described by Mandel and Higa (J. Mol. Biol. 53 (1970), 159-162). The selection of the transformed cells is undertaken by spreading on an L. Agar medium (10 g Bacto tryptone, 5 g YE and 10 g NaCl, 16 g Difco agar per litre of H₂O) containing 100 μ g/ml ampicillin. In this manner it is possible to isolate only those cells containing the plasmid with the gene of ampicillin resistance (Amp^R).

From an Amp^R clone there is extracted and purified, according to the well known methods (cf.: Rodriguez et al. in "Recombinant DNA Techniques: an introduction" (1983) Addison-Wesley Publishing Company), the plasmid pSM116, the restriction map of which is reproduced in Figure 5.

The plasmid pSM116 when compared with the plasmid pSM110 by means of electrophoresis on an agarose gel differs from the latter by the presence of a BamHI site in place of the PvuII site.

EXAMPLE 2

Formation of the plasmid pSM131

2 μ g of the plasmid pSM116, obtained as stated in example 1, are diluted in 50 μ l of solution A and digested with 2 U of restriction enzyme PstI (BIOLABS).

At the end of the digestion reaction 50 μ l of H₂O and 100 μ l of phenol are added to the mixture.

The phenol is then extracted from the aqueous phase with an equal volume of ether, and DNA is precipitated by addition of 1/10 of the volume of a 3M solution of sodium acetate (pH 5.5) and 2.5 volumes of 95% ethanol, at a temperature of -70°C for 10 minutes.

The DNA precipitated in this manner is separated from the solution by centrifuging at 10,000 revolutions per minute for 10 minutes and is then resuspended in 30 μ l of Solution A in the presence of 0.6 U of enzyme Bal 31.

The digestion reaction is conducted at 23°C for 6 minutes. The DNA linearized in this manner is precipitated and separated from the reaction mixture in the manner as stated above.

The plasmid DNA is resuspended in 20 μ l of H₂O and 1 μ l of the said aqueous solution (50 ng of DNA) is bonded to 50 ng of DNA of the XhoI linker (Wortington) in the presence of 0.1 U of T4 ligase, after suspension in 10 μ l of solution B in the manner stated in example 1.

The XhoI linkers had previously been phosphorylated at the terminal 5' position according to the description in "Molecular Cloning. A laboratory manu-

al" (Ed. T. Maniatis, E.F. Fritsch, J. Sambrook (1982) Cold Spring Harbor Laboratory).

The DNA of the XhoI linkers, the sequence of which is the following:

TCTCGAGA

AGAGCTCT, when it polymerizes, generates the recognition site for the enzyme BglII

TCTCGAGATCTCGAGA

AGAGCTCTAGAGCTCT

BglII

If at least two XhoI linkers are then bonded to the DNA of the plasmid pSM116, cut with PstI and treated with Bal31, the result will be the formation of a construct which will exhibit two new restriction sites: XhoI and BglII. 10 μ l of the ligase mixture are used to transform cells of *E. coli* HB101 rendered competent as described previously (Example 1). The cells are selected for tetracycline resistance and ampicillin sensitivity by spreading on an L. agar medium containing 20 μ g/ml of tetracycline.

The plasmids are extracted from the 44 Tc^R and Amp^S clones by the rapid procedure.

The plasmid DNAs obtained in this manner are digested with 1 U of enzyme XhoI after suspension in 20 μ l of solution A, for the purpose of verifying the completion of the insertion of the linker.

After analysis on an agarose gel (7%) the plasmids linearized with XhoI and with reduced lengths as compared with pSM116 were subsequently analysed for the purpose of verifying the presence of the BglII restriction site. The plasmid containing both the BglII and the XhoI restriction site and having a reduced length as compared with pSM116 was designated pSM131.

The regions in proximity to the BglII site of the BglII-BamHI fragments of the plasmid pSM131 are sequenced by the method of Maxam and Gilbert (Methods in Enzymology, vol. 65, pp. 499-560 (1980)).

Figure 6 shows the restriction map of the plasmid pSM131 and the precise arrangement of the two XhoI linkers. The said linkers are situated at a distance of 69 bases from the stop codon (TAA) of β -lactamase (position 3224 of pBR322) and at 532 bases from its start codon (ATG) (position 3618 of pBR322), thus indicating that the enzyme Bal31 has deleted approximately 390 pair of bases (bp) from the PstI site towards the origin of replication and only 5 bp from the PstI site towards the EcoRI site. Accordingly, the plasmid pSM131 exhibits the expected characteristics, possessing the XhoI and BglII restriction sites a short distance downstream of the origin of replication of pBR322.

EXAMPLE 3

Cloning of the terminator of the phage fd in pUC8 and formation of the plasmid pSM138.

1 μ g of the plasmid pUC8 (Boehringer-Mannheim) and 1 μ g of the plasmid pLUB3 of *E. coli* (R. Gentz et al.: PNAS 78, 4936 (1981)) are each digested with 1 U of restriction enzyme HindIII (BIOLABS) after suspension in 20 μ l of solution A.

Whereas the pUC8 is linearized from the cut with HindIII, the pLUB3 is cut into two segments, the smaller of which, of approximately 352 bp, contains the terminating sequence of the phage fd.

The plasmid DNAs are precipitated and separated from the reaction mixtures in the manner stated previously.

60 ng of pLUB3-HindIII and 15 ng of pUC8-HindIII are suspended in 10 µl of solution B and bonded together in the presence of 0.1 U of T4 ligase.

2 µl of ligase mixture are utilized for the purpose of transforming suitable cells of *E. coli* JM83 (BRL).

The transformants are selected for resistance to ampicillin by spreading on an L-agar medium (DIFCO) containing 100 µg/ml of ampicillin and the indicator X-gal in the manner described by J. Messing (Methods in Enz. vol. 101 (1983) pp. 20-78).

12 white colonies are obtained in this manner, from which the plasmids are extracted by the rapid procedure. 8 of the said plasmids are composed of the DNA of pUC8 and of the HindIII fragment of pLUB3 containing the terminator of the phage fd.

In the HindIII fragment of the terminator a BamHI site is located at a distance of only 4 bases from one of the HindIII ends. Since a BamHI site is also present on the pUC8 at a distance of a few bases from the HindIII site, it is possible to isolate the terminator of the hybrid plasmids obtained in this manner by digestion with the enzyme BamHI, provided that the orientation of the HindIII fragment of the terminator in pUC8 is such that the two BamHI sites are in distal positions.

The hybrid plasmids are then digested with BamHI and the DNA separated on 7.5% acrylamide gel.

After staining with ethidium bromide and visualization of the DNA under UV light, it is observed that two of the eight plasmids analysed exhibited, after the cut with BamHI, two segments of DNA, one of approximately 350 bp and the other having the length of pUC8.

The restriction map of one of these plasmids, which are designated pSM138, is reproduced in Figure 7.

EXAMPLE 4

Insertion of the terminator into the plasmid pSM131 and formation of the plasmid pSM137.

1 µg of pSM131 and 3.5 µg of pSM138 obtained as stated in examples 2 and 3 are digested, respectively, with 1 U of enzyme BglII and 3.5 U of BamHI, after suspension in 20 µl of solution A.

At the end of the reaction, the enzymes are inactivated at 65°C for 10 minutes.

2 µl of the reaction mixtures are added to 20 µl of solution B and bonded with 0.1 U of T4 ligase.

Since the enzymes BamHI and BglII generate, after cutting of the DNA, cohesive ends which can be bonded together, it is possible to obtain hybrid molecules formed from the pSM131 and from the small BamHI fragment of the pSM138 containing the terminator of the phage fd.

2 µl of the ligase mixture are used for the purpose of transforming suitable cells of *E. coli* HB101.

The transformants are then selected on plates of L agar containing 20 µg/ml tetracycline.

Among the Tc^R clones obtained in this manner, one was isolated, bearing the hybrid plasmid pSM137, the restriction map of which is reproduced in Figure 8.

The said plasmid is composed of the pSM131 and of the small BamHI fragment of pSM138 containing the terminator of the phage fd which is thus located before (in the anticlockwise sense) the origin of replication of the plasmid.

EXAMPLE 5

Introduction of the HindIII site after the structural gene of β-lactamase, and formation of the plasmid pSM141.

1 µg of the phage M13 mp9 having a double helix (RF) and 1 µg of pSM116, obtained as stated in example 1, are digested, respectively, with 1 U of enzyme EcoRI and 1 U of enzyme BamHI, after suspension in 20 µl of reaction mixture A, under the conditions recommended by the supplier company Biolabs. After inactivation of the enzyme at 65° for 10 minutes, 1 µl of each of each of the two mixtures are bonded together, after suspension in 20 µl of reaction mixture B, in the presence of 0.1 U of T4 ligase.

5 µl of ligase mixture are then used for the purpose of transforming suitable cells of *E. coli* JM83, selection being carried out by resistance to ampicillin. The DNA (RF) of the phages is prepared from twelve of the white plaques obtained in this manner, as described by M. Zoller and M. Smith (Nucl. Ac. Res. 10 (1982) 6487-6500).

The DNAs are then digested with the enzymes BamHI and EcoRI as stated previously, and the samples are separated on 0.8% agarose gel.

Two of the hybrid molecules obtained in this manner prove to be formed by the DNA of the phage M13 mp9 and by the segment of 2000 bp of the plasmid pSM116 bearing the gene of β-lactamase.

From one of the two plaques there is then prepared the single-helix from of the recombinant phage DNA, purified as described by Zoller and Smith (Nucl. Ac. Research 10 (1982) 6487-6500).

A synthetic oligonucleotide of 18 bases was then designed to be complementary to the DNA region which follows the end of the gene of β-lactamase, apart from one base, and the sequence of which is the following:

5' TAACTGTCAGACCAAGTTTACTCATATAT-3'
GTCTGGTTC AATGAGTA 5' synthetic primer

G
HindIII

The single-helix DNA of the recombinant phage, the universal primer TCCAGTCACGACGT (Biolabs) and the synthetic oligonucleotide (synthesized by Creative Biomolecules - San Francisco - Ca) are used for the experimental investigation of in vitro mutation as described by K. Norris et

al. (Nucl. Ac. Research II (1983) 5103-5112), for the purpose of positioning a HindIII site after the end of the gene of β -lactamase.

For the purpose of the *in vitro* polymerization and ligation reaction, the reaction mixture is treated with 3 U of BamHI and 3 U of EcoRI under the conditions recommended by the supplier company (Biolabs). The DNA is then separated on 0.8% agarose gel, and the EcoRI-BamHI fragment of 2000 bp, which is visible under UV light after staining with ethidium bromide, is eluted in the manner described in Molecular Cloning. A laboratory Manual, Ed. T. Maniatis, E.F. Fritsch, J. Sambrook (1982) Cold Spring Harbor Laboratory.

100 ng of the segment of DNA eluted in this manner and 50 ng of the EcoRI-BamHI fragment, of approximately 350 bp, of the plasmid pSM116, recovered from 7.5% acrylamide gel according to the procedure described by Maxam and Gilbert in Methods in Enzymology vol. 65 (1980) 526-527, are bonded in the presence of 0.1 U of T4 ligase, after suspension in 50 μ l of reaction mixture B 10 μ l of ligase mixture are used for the purpose of transforming suitable cells of *E. coli* HB101, selecting the transformants for resistance to ampicillin (100 μ g/ml).

The plasmid DNA is prepared from four Amp^R clones according to the rapid procedure.

The DNA preparations are then treated with 1 U of enzyme HindIII in 30 μ l of reaction mixture A.

In the case of complete mutation, it was expected that the cutting with the said enzyme would generate two fragments of DNA, one of 860 bp and the other of 1600 bp.

The analysis, on 1% agarose gel, of the four samples obtained as stated above indicated that two of these exhibited the expected characteristics.

One of the said plasmids was designated pSM141.

Its restriction map and the steps required for the preparation thereof are reproduced in Figure 9.

EXAMPLE 6

Formation of the plasmid pSM142

1 μ g of the plasmid pSM141 and 1 μ g of the plasmid pSM137, which was obtained as stated in example 4, are each digested with 1 U of enzyme HindIII, after suspension in 20 μ l of solution A.

The enzyme HindIII cuts the plasmid pSM141 at two points, generating two fragments, one of 830 bp and the other of 1600 bp. The pSM137 also exhibits two HindIII sites, and thus the digestion thereof gives rise to a fragment of 550 bp and to one of 3500 bp (Figure 8).

1 μ l of each reaction mixture is added to 18 μ l of solution B containing 0.1 U of enzyme T4 ligase. The ligation reaction is carried out at 14°C for 14 hours.

After inactivation of the enzyme at 65° for 10 minutes, 5 μ l of ligase mixture are used for the purpose of transforming suitable cells of *E. coli* HB101.

The transformants are selected for resistance to ampicillin and to tetracycline, by spreading on an L agar medium containing 100 μ g/ml of ampicillin and 20 μ g/ml of tetracycline.

From a Tc^R, Amp^R clone there is isolated by the rapid procedure a plasmid formed by the fragment of approximately 830 bp of the pS141 and by the fragment of approximately 3500 bp of the pSM137.

The plasmid, the restriction map of which is represented in Figure 10, was designated pSM142.

EXAMPLE 7

Formation of the plasmid pSM143

1 μ g of the plasmid pSM142 and 1 μ g of the plasmid pSM112, which was obtained as stated in Italian Patent Application A/8 423 992 (EP-A 186 631) and represented in Figure 11, are suspended in 20 μ l of solution A and simultaneously cut with 1 U of enzyme BamHI and 1 U of enzyme EcoRI at 37°C for 1 hour.

After inactivation of the enzymes at 65° for 10 minutes, 1 μ l of the reaction mixture is used for the ligation reaction in the presence of T4 ligase as previously described.

The entire ligase mixture is then employed for the purpose of transforming suitable cells of *E. coli* HB101, selecting the transformants for resistance to ampicillin and to kanamycin on plates of L agar containing 100 μ g/ml of ampicillin and 15 μ g/ml of kanamycin.

From an Amp^R and Km^R clone there is isolated by the rapid procedure the plasmid pSM143, the restriction map of which is reproduced in Figure 2.

The pSM143 is composed of the 4950 bp fragment of the pSM112 and of the 2400 bp fragment of the pSM142.

The plasmid pSM143 is then employed for the purpose of transforming cells of *B. subtilis* SMS003 NRLL B 15897, which are rendered competent as described by Contente and Dubnau (Mol. Gen. Genet. 167 (1979) 251-258).

The selection of the transformants is carried out by spreading on tryptose blood agar base (TBAB) medium from DIFCO containing 5 μ g/ml of kanamycin.

Plasmids identical to pSM143 were isolated from the Km^R clones, thus indicating that the said plasmid was maintained in a stable condition in cells of *B. subtilis*.

EXAMPLE 8

Formation of the plasmid pSM146

1 μ g of the plasmid pSM143 is digested simultaneously with 1 U of enzyme SstI and 1 U of enzyme XbaI in 20 μ l of solution A at 37°C for 1 hour.

After inactivation of the enzymes at 65° for 10 minutes, 1 μ l of the reaction mixture is added to 19 μ l of the solution B containing 0.1 U of T4 ligase and 50 ng of the fragment of synthetic DNA, the sequence of which is reproduced in Figure 3.

The ligation reaction is carried out at 14°C for 14 hours.

After inactivation of the enzyme at 65° for 10 minutes, 5 μ l of the ligase mixture are used for the purpose of transforming suitable cells of *E. coli* HB101.

The selection of the transformants is then undertaken on plates of L agar containing 100 µg/ml of ampicillin. From an Amp^R clone there is isolated by the rapid procedure a plasmid in which the fragment SstI-XbaI of 740 bp of the pSM143 was replaced by the segment of synthetic DNA of 15 bp + 8 single bases. The new plasmid is designated pSM146, and its restriction map is reproduced in Figure 3.

EXAMPLE 9

Formation of the plasmid pSM147

The procedure is carried out as in example 8 above, using in the ligase reaction 50 ng of a segment of synthetic DNA whose sequence is reproduced in Figure 4.

The plasmid isolated from an Amp^R clone, designated pSM147, exhibits, in comparison with the pSM143, the replacement of the SstI-XbaI fragment by the synthetic segment of 24 bp + 8 single bases.

The restriction map of the plasmid pSM147 is reproduced in Figure 4.

EXAMPLE 10

Determination of the β-lactamase activity of pSM143, pSM146 and pSM147.

Cells of *E. coli* HB101 and *B. subtilis* BGSC 1A246, containing the plasmids pSM143, pSM146, pSM147 and pSM119 (comparison), are cultivated in conical flasks having a capacity of 250 ml, each containing 25 ml of culture medium previously sterilized at 120° for 15 minutes.

B. subtilis is cultivated in SMS (Spitzen Minimal Safts) medium to which is added glucose (0.5%), tryptophan (50 µg/ml) and trace elements.

E. coli is cultivated in LB. (DIFCO) medium.

The conical flasks are incubated, with agitation, at 37°C for 6 hours.

The production of β-lactamase is determined, for *E. coli*, in the periplasmic specimens obtained as described by A. Nicolaidis et al. (*J. Bacteriol.* 135 (1978), 178) and, for *B. subtilis*, both in the supernatant liquid and in the sonicated cells. In order to prepare the sonicated cells, 5 ml of the *B. subtilis* culture are centrifuged at 5000 rpm for 10 minutes. The cells obtained in this manner are suspended in 5 ml of solution containing 30 mM tris. HCl, pH 7.2, and 50 mM NaCl and centrifuged again at 5000 rpm for 10 minutes.

The cells are then suspended in 5 ml of 100 mM phosphate buffer, pH 7.0, and treated 3 times (for 1 minute each time) with an MSE sonicator, Soniprep model 150, operating at maximum intensity.

The β-lactamase activity is measured with Nitrocefin as described by O'Callaghan et al. (*Antimicrob. Ag. Chemother.* 1 (1972) 283-288).

1 unit of β-lactamase is defined as the quantity of enzyme required to hydrolyse 1 nanomole of substrate per minute at 37°C.

Table I shows the figures for β-lactamase activi-

ty which were determined in the various cultures of *B. subtilis* BGSC 1A246 and *E. coli* HB101.

TABLE I

β-LACTAMASE ACTIVITY (U/ml)

PLASMID	E. COLI HB101	B. subtilis BGSC 1A246
pSM119	1300 U/ml	* 380 U/ml
pSM143	2000 U/ml	* 700 U/ml
pSM146	12000 U/ml	—
pSM147	85000 U/ml	—

* Total of the activity determined in the supernatant liquid and in the sonicated cells.

Claims

1. Plasmid vectors for expression in *Escherichia coli* and *Bacillus subtilis* of a heterologous protein wherein the gene which codes for said protein is placed under the control of the terminator sequence of the phage fd of *Escherichia coli* and of the pE194 erythromycin promoter modified by replacing the SstI-XbaI fragment of 740 pairs of bases containing the -35 region of the said promoter with a segment of synthetic DNA having a number of pair bases from 13 to 22 and 8 single bases.

2. Plasmid vector pSM146 according to claim 1, wherein the synthetic DNA segment is characterized by the following sequence of bases:

5' CTGTTTTTTGTCAAT 3'

3' TCGAGACAAAAACAGTTAGATC 5'

3. Plasmid vector pSM147 according to claim 1, wherein the synthetic DNA segment is characterized by the following sequence of bases:

5' CTGTTTTTTGTCAACAACTTTTTT 3'

3' TCGAGACAAAAACAGTTGTTGAAAAA-
GATC 5'

4. Plasmid vector according to claims 1 to 3, wherein the gene of the heterologous protein is that of β-lactamase.

5. Process for the construction of the plasmid vectors according to claims 1 to 3 from the plasmid vector pSM143 characterized in that:

a) said plasmid pSM143 is digested with the restriction enzymes SstI and XbaI which cut in the unique sites SstI and XbaI of the pE194 erythromycin promoter and

b) the SstI-XbaI fragment of 6610 pair of bases is ligated, in the presence of DNA T4 ligase, to the synthetic DNA segment having the sequence according to claims 2 and 3.

6. Plasmid vector pSM143 which comprises the origin of replication of pBR322 and pUB110 which allow its propagation in *Escherichia coli* and *Bacillus subtilis*, the kanamycin resistance selectable marker functioning in both microorganisms, the β-lactamase structural gene located between the terminator of the phage fd and the promoter of the pE194 erythromycin gene and the unique restriction sites XbaI, SstI, EcoRI, HindIII, XhoI.

7. Process for the construction of the plasmid

vector pSM143 according to claim 6, characterized in that:

- a) the plasmid pSM110 is modified by replacing the PvuII site with the BamHI site, and the plasmid pSM116 is isolated,
 - b) there are located within the plasmid pSM116, downstream of the origin of replication of PBR322 two restriction sites XhoI and BglII which are useful for cloning, and the plasmid pSM131 is isolated,
 - c) the HindIII fragment bearing the terminator of the phage fd of E. coli is inserted in the plasmid pUC8, and the plasmid pSM138 is isolated,
 - d) the terminator of the phage fd of the plasmid pSM138 is isolated, following digestion with the restriction enzyme BamHI,
 - e) the BamHI fragment containing the terminator of the phage fd is inserted in the BglII site of the plasmid pSM131, and the plasmid pSM137 is isolated,
 - f) a HindIII site is introduced downstream of the structural gene of the β -lactamase by means of in vitro mutation of the plasmid pSM116, and the plasmid pSM141 is isolated,
 - g) there are bonded, in the presence of DNA T4 ligase, the HindIII fragment of the pSM141 containing the gene of β -lactamase and the HindIII fragment of 3500 pair of bases of the plasmid pSM137, which were obtained following digestion of the two plasmids, and the plasmid pSM142 is isolated,
 - h) the BamHI-EcoRI fragment of the plasmid pSM142 is bonded, in the presence of DNA T4 ligase, with the plasmid pSM112, following digestion of the two plasmids with the restriction enzymes BamHI and EcoRI, and the plasmid pSM143 is isolated.
8. Process for the production of β -lactamase, characterized in that:
- a) the plasmid vectors pSM143, pSM146 and pSM147 are introduced into host organisms,
 - b) the said organisms transformed in this manner are isolated and
 - c) the said transformed organisms are cultivated in a liquid culture medium containing sources of carbon, sources of nitrogen and trace elements.
9. Process according to claim 8, wherein the host organisms are Escherichia coli HB101 and Bacillus subtilis BGSC 1A246.
10. Escherichia coli (pSM143) ATCC 53038
 11. Escherichia coli (pSM146) ATCC 53039
 12. Escherichia coli (pSM147) ATCC 53040
 13. Escherichia coli (pSM138) ATCC 53037

Patentansprüche

1. Plasmidvektoren zur Expression eines heterologen Proteins in Escherichia coli und Bacillus subtilis, wobei das für dieses Protein kodierende Gen unter die Regelung der Terminatorsequenz des Phagen fd von Escherichia coli und des pE194-Erythromycinpromoters gesetzt wird, welcher Promoter durch Ersatz des SstI-XbaI-Fragmentes von 740 Basenpaaren, welches Fragment den -35-Bereich dieses Promoters enthält, durch ein syntheti-

sches DNA-Segment modifiziert worden ist, welches Segment eine Anzahl von 13 bis 22 Basenpaaren und 8 Einzelbasen aufweist.

2. Plasmidvektor pSM146 nach Anspruch 1, worin das synthetische DNA-Segment durch die folgende Sequenz von Basen gekennzeichnet ist:

5' CTGTTTTTTGTCAAT 3'

3' TCGAGACAAAAAACAGTTAGATC 5'

3. Plasmidvektor pSM147 nach Anspruch 1, worin das synthetische DNA-Segment durch die folgende Sequenz von Basen gekennzeichnet ist:

5' CTGTTTTTTGTCAACAACCTTTTTT 3'

3' TCGAGACAAAAAACAGTTGTTGAAAAAATGATC 5'

4. Plasmidvektor nach einem der Ansprüche 1 bis 3, worin das Gen des heterologen Proteins jenes von β -Lactamase ist.

5. Verfahren für den Aufbau der Plasmidvektoren nach einem der Ansprüche 1 bis 3, aus dem Plasmidvektor pSM143, dadurch gekennzeichnet, daß:

a) dieses Plasmid pSM143 mit den Restriktionsenzymen SstI und XbaI digeriert wird, welche anschließend an den SstI- und XbaI-Stellen des pE194-Erythromycinpromoters schneiden; und

b) das SstI-XbaI-Fragment von 6610 Basenpaaren in Gegenwart von DNA-T4-Ligase mit dem synthetischen DNA-Segment verknüpft wird, welches die Sequenz nach den Ansprüchen 2 und 3 aufweist.

6. Plasmidvektor pSM143, welcher den Ausgangsbereich für die Entstehung von Kopien von PBR322 und pUB110 enthält, welche letzteren dessen Vermehrung in Escherichia coli und Bacillus subtilis ermöglichen, wobei der für Kanamycin-Resistenz wählbare Marker in beiden Mikroorganismen funktioniert, und wobei das β -Lactamase-Strukturgen zwischen dem Terminator des Phagen fd und dem Promoter des pE194-Erythromycingens sowie den ausschließlichen Restriktionsstellen XbaI, SstI, EcoRI, HindIII und XhoI angeordnet ist.

7. Verfahren für den Aufbau des Plasmidvektors pSM143 nach Anspruch 6, dadurch gekennzeichnet, daß:

a) das Plasmid pSM110 dadurch modifiziert wird, daß man die PvuII-Stelle durch die BamHI-Stelle ersetzt und das Plasmid pSM116 isoliert;

b) in dem Plasmid pSM116 - stromabwärts von dem Ausgangsbereich für die Entstehung von Kopien des pBR322 - zwei Restriktionsstellen XhoI und BglII angeordnet werden, welche für das Klonen brauchbar sind; und das Plasmid pSM131 isoliert wird;

c) das den Terminator des Phagen fd von E. coli tragende HindIII-Fragment in das Plasmid pUC8 eingesetzt wird, und das Plasmid pSM138 isoliert wird;

d) der Terminator des Phagen fd des Plasmids pSM138 im Anschluß an das Digerieren mit dem Restriktionsenzym BamHI isoliert wird;

e) das den Terminator des Phagen fd enthaltende BamHI-Fragment an der BglII-Stelle des Plasmids pSM131 eingesetzt wird und das Plasmid pSM137 isoliert wird;

f) stromabwärts von dem Strukturgen der β -

Lactamase mit Hilfe einer in-vitro-Mutation des Plasmids pSM116 eine HindIII-Stelle eingeführt wird, und das Plasmid pSM141 isoliert wird;

g) das das Gen von β -Lactamase enthaltende HindIII-Fragment des pSM141 und das HindIII-Fragment von 3500 Basenpaaren des Plasmids pSM137, welche nach dem Digerieren der zwei Plasmide erhalten worden waren, in Gegenwart von DNA-T4-Ligase miteinander verbunden werden, und das Plasmid pSM142 isoliert wird;

h) das BamHI-EcoRI-Fragment des Plasmids pSM142 - in Gegenwart von DNA-T4-Ligase - mit dem Plasmid pSM112 verbunden wird, und zwar im Anschluß an das Digerieren der zwei Plasmide mit den Restriktionsenzymen BamHI und EcoRI, und das Plasmid pSM143 isoliert wird.

8. Verfahren zur Herstellung von β -Lactamase, dadurch gekennzeichnet, daß:

a) die Plasmidvektoren pSM143, pSM146 und pSM147 in Wirtsorganismen eingeführt werden;

b) die auf diese Weise transformierten Organismen isoliert werden; und

c) diese transformierten Organismen in einem flüssigen Kulturmedium kultiviert werden, welches Quellen für Kohlenstoff, Quellen für Stickstoff und Spurenelemente enthält.

9. Verfahren nach Anspruch 8, wobei die Wirtsorganismen *Escherichia coli* HB101 und *Bacillus subtilis* BGSC 1A246 sind.

10. *Escherichia coli* (pSM143) ATCC 53038

11. *Escherichia coli* (pSM146) ATCC 53039

12. *Escherichia coli* (pSM147) ATCC 53040

13. *Escherichia coli* (pSM138) ATCC 53037.

Revendications

1. Plasmides vecteurs pour l'expression d'une protéine hétérologue dans *Escherichia coli* et *Bacillus subtilis*, dans lesquels le gène qui code pour ladite protéine est placé sous le contrôle de la séquence terminateur du phage fd d'*Escherichia coli* et du promoteur érythromycine de pE194 modifié par remplacement du fragment SstI-XbaI de 740 paires de bases, contenant la région -35 dudit promoteur, par un segment d'ADN synthétique comportant un nombre de 13 à 22 paires de bases et 8 bases non apparées.

2. Plasmide vecteur pSM146 conforme à la revendication 1, dans lequel le segment d'ADN synthétique est caractérisé par la séquence suivante de bases:

5' CTGTTTTTTTGCAAT 3'

3' TCGAGACAAAAACAGTTAGATC 5'

3. Plasmide vecteur pSM147 conforme à la revendication 1, dans lequel le segment d'ADN synthétique est caractérisé par la séquence suivante de bases:

5' CTGTTTTTTTGCAACAACCTTTTTT 3'

3' TCGAGACAAAAACAGTTGTTGAAAAAAGATC 5'

4. Plasmid vecteur conforme aux revendications 1 à 3, dans lequel le gène de la protéine hétérologue est celui de la β -lactamase.

5. Procédé pour la construction des plasmides

vecteurs conformes aux revendications 1 à 3 à partir du plasmide vecteur pSM143, caractérisé en ce que:

a) ledit plasmide pSM143 est digéré par les enzymes de restriction SstI et XbaI qui le coupe au niveau des sites uniques SstI et XbaI du promoteur érythromycine de pE194, et

b) le fragment SstI-XbaI de 6610 paires de bases est lié, en présence d'ADN ligase de T4, au segment d'ADN synthétique présentant la séquence conforme à la revendication 2 ou 3.

6. Plasmide vecteur pSM143, qui comporte les origines de répllication de pBR322 et de pUB110 qui permettent sa prolifération dans *Escherichia coli* et dans *Bacillus subtilis*, le marquer de sélection par résistance à la kanamycine, fonctionnant dans les deux micro-organismes, le gène structural de la β -lactamase, situé entre le terminateur du phage fd et le promoteur du gène d'érythromycine de pE194, et les sites uniques de restriction XbaI, SstI, EcoRI, HindIII, et XhoI.

7. Procédé pour la construction du plasmide vecteur pSM143 conforme à la revendication 6, caractérisé en ce que:

a) on modifie le plasmide pSM110 en remplaçant le site PvuII par le site BamHI, et on isole le plasmide pSM116;

b) on place dans le plasmide pSM116, en aval de l'origine de répllication de pBR322, deux sites de restriction XhoI et BglII, utiles pour le clonage, et on isole le plasmide pSM131;

c) on insère dans le plasmide pUC8 le fragment HindIII, portant le terminateur du phage fd de *E. coli*, et on isole le plasmide pSM138;

d) on isole le terminateur du phage fd du plasmide pSM138, à la suite d'une digestion par l'enzyme de restriction BamHI;

e) on insère le fragment BamHI, contenant le terminateur du phage fd, dans le site BglII du plasmide pSM131, et on isole le plasmide pSM137;

f) on introduit un site HindIII en aval du gène de structure de la β -lactamase, au moyen d'une mutation in vitro du plasmide pSM116, et on isole le plasmide pSM141;

g) on lie, en présence d'ADN ligase de T4, le fragment HindIII de pSM141, contenant le gène de la β -lactamase, et le fragment HindIII de 3500 paires de bases du plasmide pSM137, fragments que l'on obtient à la suite de la digestion des deux plasmides, et on isole le plasmide pSM142;

h) on lie le fragment BamHI-EcoRI du plasmide pSM142, en présence d'ADN ligase de T4, avec le plasmide pSM112, à la suite de la digestion des deux plasmides par les enzymes de restriction BamHI et EcoRI, et on isole le plasmide pSM143.

8. Procédé pour la production de β -lactamase, caractérisé en ce que:

a) on introduit les plasmides vecteurs pSM143, pSM146 et pSM147 dans des organismes hôtes,

b) on isole lesdits organismes-transformés de cette manière, et

c) on cultive lesdits organismes transformés dans un milieu liquide de culture, qui contient des sources de carbone, des sources d'azote et des oligo-éléments.

9. Procédé conforme à la revendication 9, dans lequel les organismes hôtes sont *Escherichia coli* HB101 et *Bacillus subtilis* BGSC 1A246.

10. *Escherichia coli* (pSM143) ATCC 53038.

11. *Escherichia coli* (pSM146) ATCC 53039.

12. *Escherichia coli* (pSM147) ATCC 53040.

13. *Escherichia coli* (pSM138) ATCC 53037.

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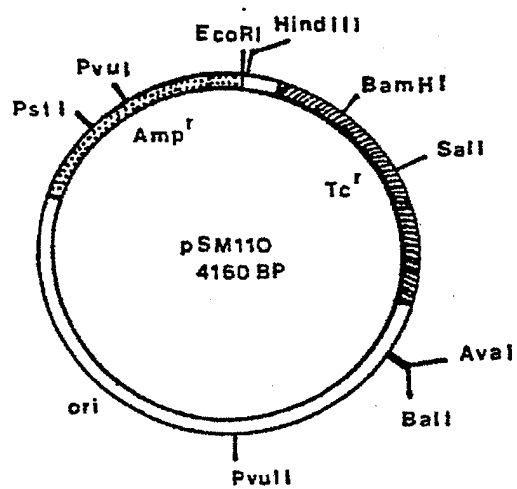


FIG. 1

FIG. 2

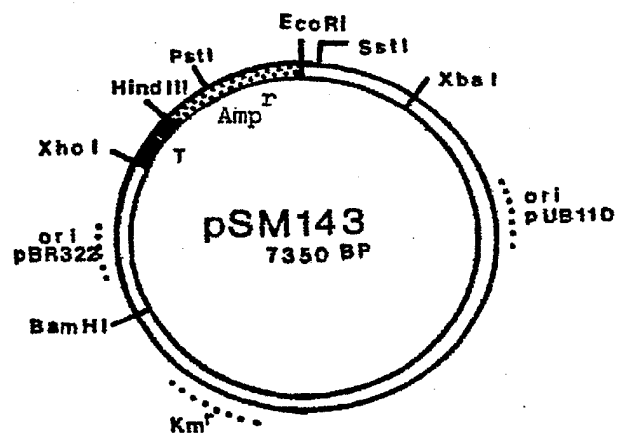
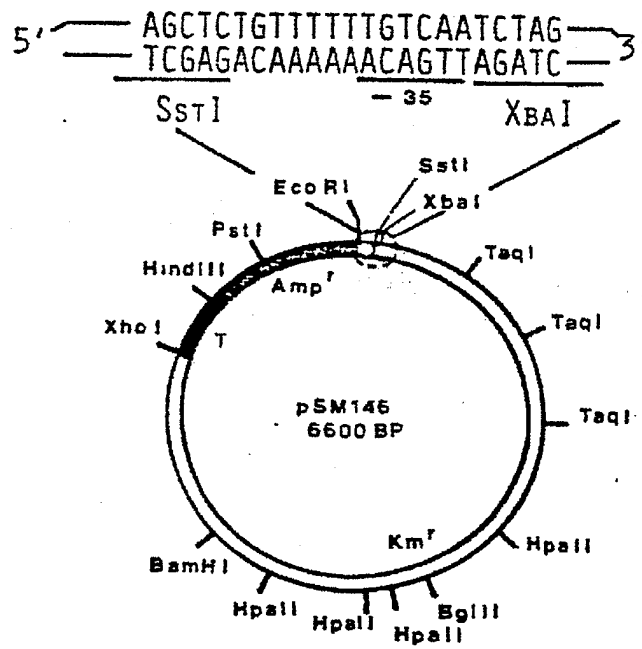


FIG. 3



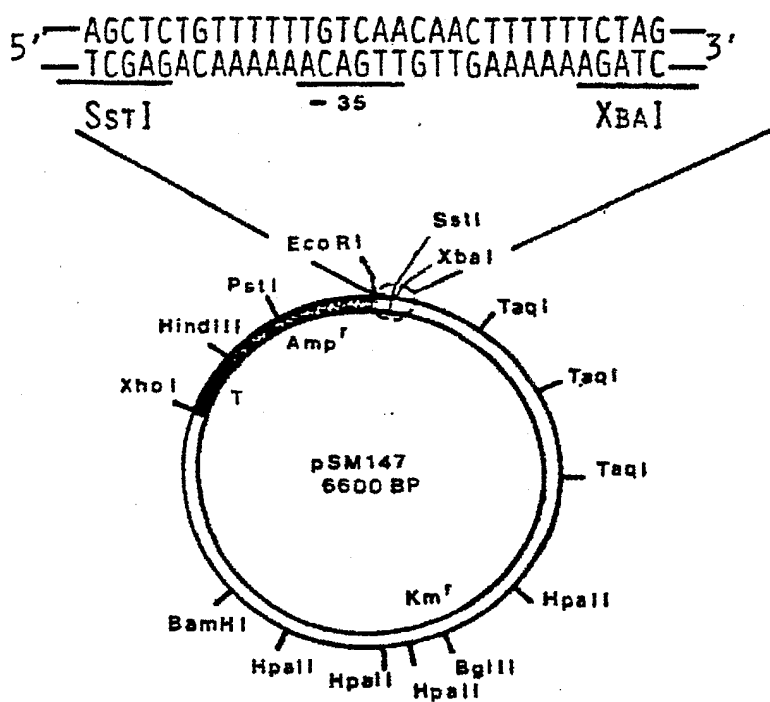


FIG. 4

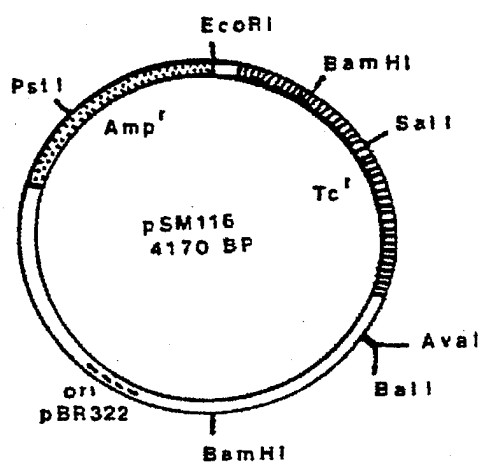


FIG. 5

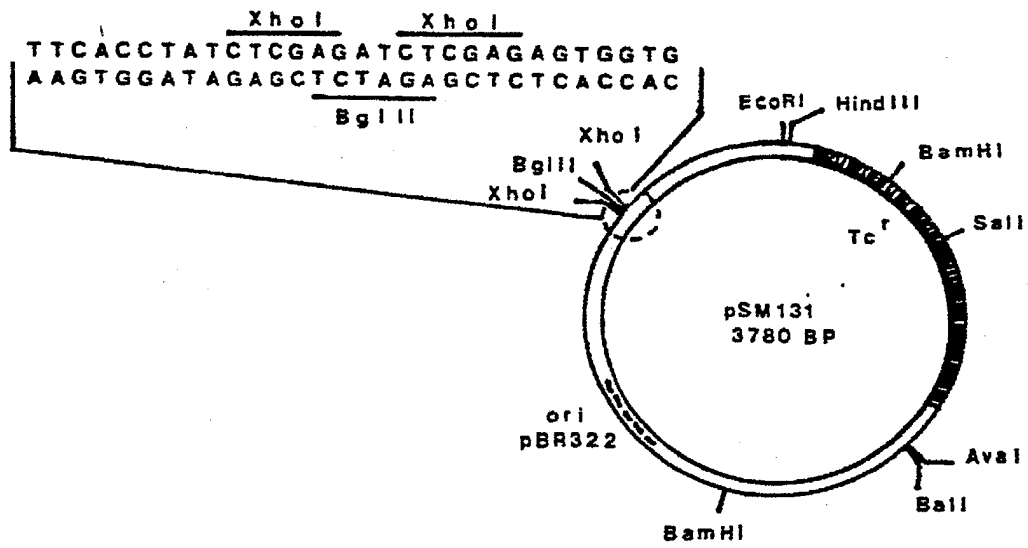


FIG. 6

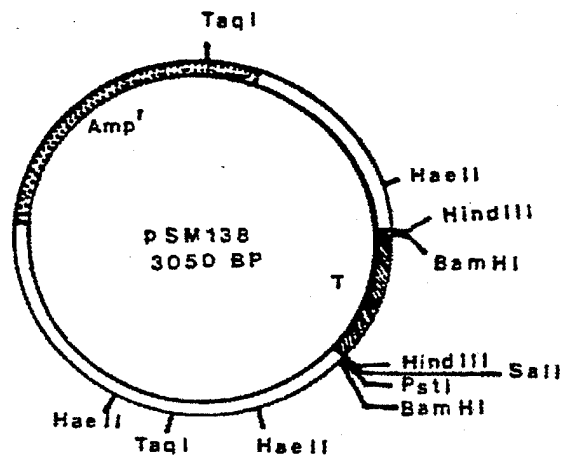


FIG. 7

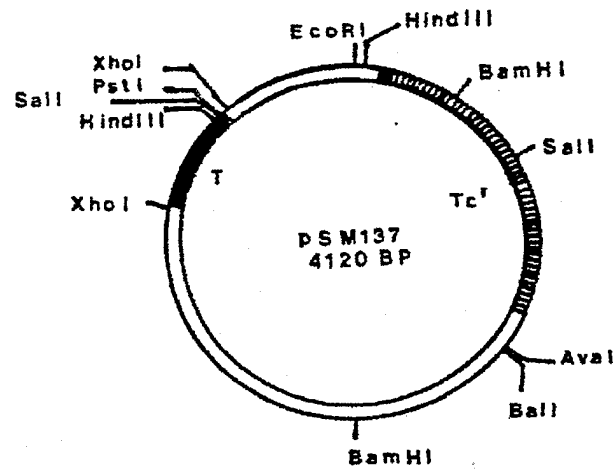


FIG. 8

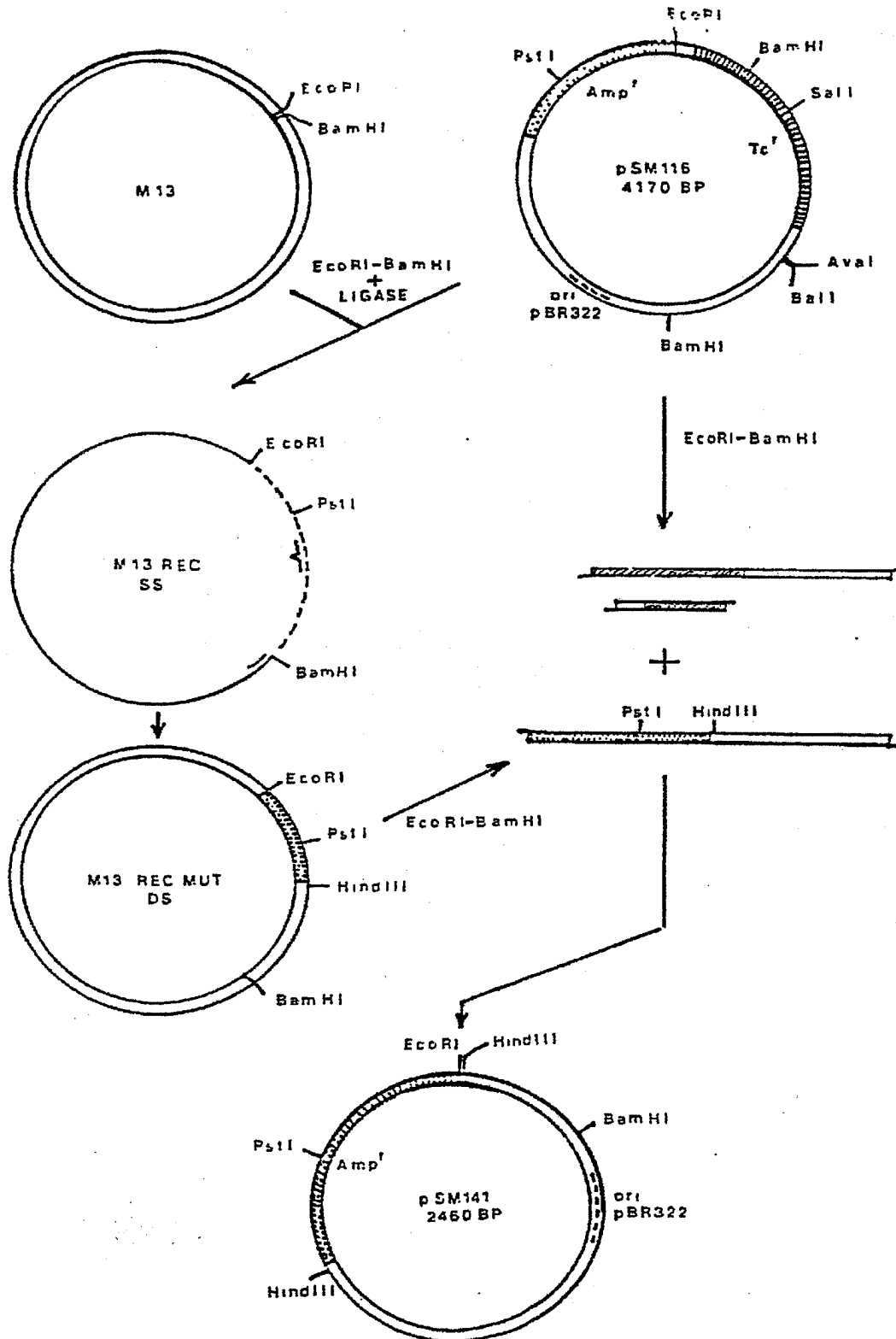


FIG. 9

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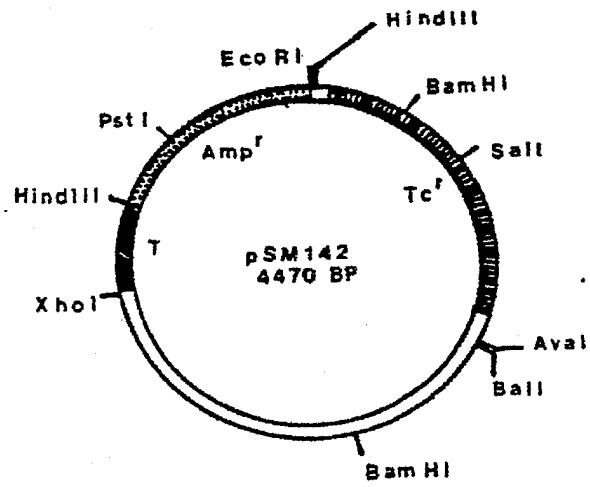
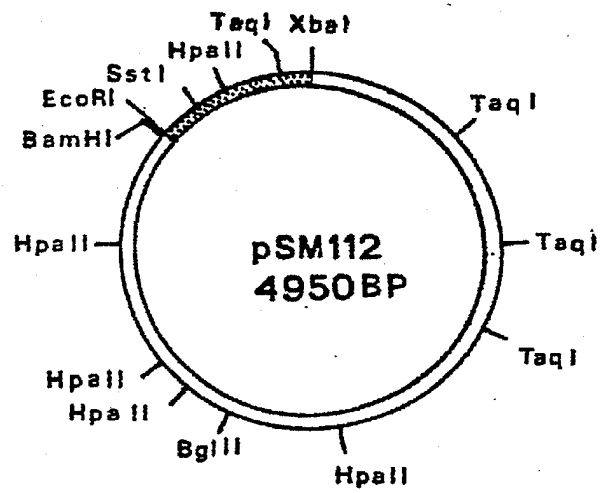


FIG. 10

FIG. 11



==== pUB110

----- pE194